

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS

Event-specific Method for the Quantification of Soybean FG72 Using Real-time PCR

Validation Report and
Validated Method

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Event-specific Method for the Quantification of Soybean FG72 Validation Report

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**European Union Reference Laboratory for GM Food and Feed
Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

Executive Summary

In line with its mandate¹ the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific PCR method for detecting and quantifying soybean event FG72 (unique identifier MST-FGØ72-2). The validation study was conducted according to the EU-RL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and internationally accepted guidelines², involving 12 laboratories.

In accordance with current EU legislation¹, Bayer CropScience has provided the detection method and the samples (genomic DNA from soybean seeds harbouring the FG72 event as positive control DNA, genomic DNA from conventional soybean seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL and the ENGL detailing the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

¹ Regulation (EC) No 1829/2003 of 22 September 2003 “on genetically modified food and feed” and with Regulation (EC) No 641/2004 of 6 April 2004 “on detailed rules for the implementation of Regulation (EC) No 1829/2003”,

² The IUPAC “Protocol for the design, conduct and interpretation of method-performance studies” (Horwitz, 1995); and ISO 5725 (1994).

Quality assurance

The EU-RL GMFF is ISO 9001:2008 certified (certificate number: CH-32232) and ISO 17025:2005 accredited [certificate number: ACCREDIA 1172, (Flexible Scope for DNA extraction and qualitative/quantitative PCR) - Accredited tests are available at http://www.accredia.it/accredia_labsearch.jsp?ID_LINK=293&area=7].

The original version of the document containing evidence of internal checks and authorisation for publication is archived within the EU-RL GMFF quality system.

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1. Summary

In line with Regulation (EC) No 1829/2003, Bayer CropScience provided the EU-RL GMFF with an event specific method for the detection and quantification of soybean event FG72 (unique identifier MST-FGØ72-2) together with control samples.

In response to this early submission of a draft dossier (step 1), the EU-RL GMFF started the step-wise validation procedure in advance respect to the declaration of valid application by EFSA (October 2011).

The scientific assessment (step 2) focused on the method performance characteristics assessed against the ENGL method acceptance criteria³ (http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf for a summary of method acceptance criteria and method performance requirements). The scientific assessment performed for soybean event FG72 was positively concluded in July 2010.

In step 3 of the validation procedure (Experimental testing), the EU-RL GMFF verified the purity of the control samples provided, conducted the in-house testing of samples and methods. The positive control DNA - submitted in accordance with Art 5(3)(j) and Article 17(3)(j) of Reg.(EC) No 1829/2003 – was found contaminated with DNA not related to the specific application. Further to request for replacement of positive control sample, its reception and testing, and following a scientific assessment the sample was considered acceptable (July 2011).

The method characteristics were verified by quantifying five blind GM levels within the range 0.1%-8% on a copy number basis. The experiments were performed under repeatability conditions and demonstrated that the PCR efficiency, linearity, accuracy and precision were within the limits established by the ENGL. In addition, and in line with the requirements of Reg. (EU) No 619/2011, the EU-RL GMFF verified *i)* the zygosity ratio of the positive control sample submitted, by investigating the GM- to reference- target ratio by means of digital PCR, in order to determine the conversion factor between copy numbers and mass fractions; *ii)* the method's precision (relative repeatability standard deviation, RSDr %) at the 0.1% related to mass fraction of GM-material on fifteen replicates. Step 3 was concluded in May 2012.

The collaborative trial (step 4) was organised and took place in August/September 2011. It demonstrated that the method is well suited to analyse DNA, appropriately extracted from food or feed, with regard to identifying the presence of GM event FG72 and is therefore applicable for this purpose.

³ EURL/ENGL guidance doc "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>)

2. Summary of the EU-RL GMFF scientific assessment on applicants' data

Documentation and data provided by the applicant were evaluated by the EU-RL GMFF for compliance with the ENGL method acceptance criteria for the parameters of the calibration curves (slope, R^2 coefficient) used to quantify in three runs three test samples at different GM-levels in haploid genome copy number.

Table 1. Values of slope and R^2 obtained by the applicant

	FG72		le1	
	R^2	Slope	R^2	Slope
Run 1	1.00	-3.4	1.00	-3.3
Run 2	1.00	-3.4	0.99	-3.4
Run 3	1.00	-3.4	1.00	-3.3

Table 1 indicates that the slope and R^2 coefficient of the standard curves for the GM-system (FG72) and the soybean-specific reference system (*le1*) were within the ENGL acceptance criteria (slope between -3.1 to -3.6 and R^2 coefficient ≥ 0.98).

Table 2. Mean %, precision and trueness measured at three GM-levels by the applicant

	Test Sample Expected GMO %		
	0.08	0.9	4.5
Mean %	0.07	0.91	4.53
Precision (RSDr %)	15	5	7
Trueness (bias %)	-14	2	7

Table 2 reports precision and trueness for the three GM-levels tested by the applicant. Eighteen values for each GM-level were provided. Both parameters were within the ENGL acceptance criteria (trueness $\pm 25\%$, RSDr $\leq 25\%$ across the entire dynamic range).

3. Materials and methods

3.1 DNA extraction

A "Dellaporta-derived" protocol for DNA extraction suitable for the isolation of genomic DNA from ground soybean seeds and grains, submitted by the applicant in support of the validation of the method for detection of event soy A2704-12, was evaluated and tested by the EU-RL GMFF in order to confirm its performance characteristics. The protocol for DNA extraction and a report on testing were published in 2007 at http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_DNAExtr_report.pdf. In agreement with the ENGL position, which endorses the modularity principle (see also Annex I to Reg. (EC) No 641/2004), the EU-RL GMFF considers the above mentioned DNA extraction protocol applicable in the context of the validation of the method for analysis of event FG72 soybean given the similarity of the soybean matrix.

3.2 Method protocol for the PCR analysis

The PCR analysis method that was provided by the applicant (see the corresponding Validated Method at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) and subsequently validated by the EU-RL GMFF is an event-specific, quantitative, real-time TaqMan® PCR (Polymerase Chain Reaction) procedure for the determination of the relative content of GM event FG72 DNA to total soybean DNA. The procedure is a simplex system, in which a soybean *le1* (*lectin 1*) specific assay, and the target assay (FG72) are performed in separate wells.

For the detection of GM event FG72, a 70-bp fragment of the region spanning the 3' insert-to-plant junction in soybean FG72 is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with FAM (6-carboxyfluorescein) as reporter dye at its 5' end and MGB-NFQ at its 3' end as quencher dye.

For the relative quantification of GM event FG72, a soybean specific reference system amplifies a 102-bp fragment of *lectin1* (*le1*), a soybean endogenous gene, using *le1* gene-specific primers and a *le1* gene-specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA (carboxytetranethylrhodamine) as quencher at its 3' end.

Standard curves are generated for both the FG72 and the *le1* systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data. Thereafter, the standard curves are used to estimate the copy numbers in the test sample DNA by interpolation from the standard curves.

For relative quantification of event FG72 DNA in a test sample, the FG72 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 to obtain the percentage value ($GM\% = FG72/le1 \times 100$).

The absolute copy numbers of the calibration curve samples are calculated by dividing the sample DNA mass (nanograms) by the published average 1C value for the soybean genome (1.13 pg) (Arumuganathan & Earle, 1991). The copy number values used in the quantification, the GMO contents of the calibration samples and total DNA quantity used in PCR are listed in Table 3.

Table 3. Copy number values of the standard curve samples.

Sample code	S1	S2	S3	S4	S5
Total amount of DNA in reaction (ng)	150	37.5	9.4	2.35	0.6
Target taxon <i>le1</i> copies	132743	33186	8296	2074	519
FG72 Soybean GM copies	13274	3319	830	207	52

3.3 EU-RL GMFF experimental testing

3.3.1 Determination of the zygosity ratio in the positive control sample

Standard and test samples were prepared at the EU-RL GMFF in terms of GM DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes. Annex II of Reg. (EU) No 619/2011 requires that "when results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be translated into mass fraction in accordance with the information provided in each validation report of the EU-RL GMFF." In order to satisfy this requirement, the EU-RL GMFF conducted an experimental assessment on the zygosity (GM-target to reference target ratio) in the positive control sample that was submitted by the applicant for the preparation of standard and test items at various GM-concentrations.

The copy number of the FG72 and of the *Le1* targets in the positive control sample submitted by the applicant were determined by digital PCR (dPCR).

Digital PCR experiments were performed on the BioMark HD System using the 12.765 digital arrays (Fluidigm).

Genomic DNA was provided by the applicant as positive control sample for FG72 soy event.

Three micrograms of genomic DNA were digested at 37 °C overnight with 20 units of the four-base cutter restriction enzyme Taq I. The latter does not cleave within the annealing sites for the primers of the FG72 or *le1* amplification systems. Taq I restriction sites are located outside the respective targeted sequences. Further to digestion, the DNA was precipitated with ammonium acetate 2.5 M final and two volumes of absolute ethanol. The outcome of enzymatic digestion was

controlled by running approximately 100 nanograms of Taq I digested and 100 nanograms of undigested DNA in comparison with DNA molecular marker in 1% agarose-gel electrophoresis.

Digested template DNA was used in digital PCR experiments. Reaction mixes were prepared in a final volume of 9 µL and contained 1X TaqMan® Universal PCR Master Mix with UNG (Applied Biosystems), 1X GE sample loading reagent (Fluidigm), primers and probe, at the reaction concentrations indicated in the corresponding Validated Method (<http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>), 1 µL of DNA at a concentration of 0.5 ng/µL, suitable to avoid panel saturation after analysis (optimal between 200<positive partitions<500).

Loading of the digital chip was performed according to the manufacturer's instructions by using the IFC controller (Fluidigm). A volume of 9 µL of reaction mix was loaded into each well of which only approximately 4.6 µL were distributed into the 765 chambers (or partitions) constituting one panel. The analysis was repeated in three days; five replicates in five panels were run each day for both the GM- and reference assay, with a total number of fifteen data sets for both targets. No template controls were included. Amplification conditions were as reported in the Validated Method. Data analysis and copy number calculation was performed using the BioMark digital PCR Analysis software applying the default settings.

Calculations of mean and variances are implemented according to the procedure outlined for random variables in the Annex 4 of the ENGL guidance document 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'⁴.

3.3.2 In-house verification of the method performance against ENGL method acceptance criteria

The method performance characteristics were verified (EU-RL GMFF step 3) by quantifying on a copy number basis five blinded test samples with known GM levels, within the range 0.1%-8%. The experiments were performed on an ABI 7900 real-time platform under repeatability conditions. Test samples with GM-levels 8.0%, 3.0%, 0.9% and 0.4% were tested in two real-time PCR runs with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in an additional run. Average values of the slope and of the R² coefficient of the standard curves and method trueness and precision of quantification over the dynamic range were evaluated for compliance against the ENGL method acceptance criteria.

In order to assess the method compliance to Reg. (EU) No 619/2011, the EU-RL GMFF also implemented tests to determine the zygosity of the GM-insert in the positive control sample and estimated the method precision (RSDr) at 0.1% GM level in mass fraction on 15 replicates.

⁴ Verification of analytical methods for GMO testing when implementing interlaboratory validated methods. European Network of GMO laboratories (ENGL), 2011.
<http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf>

3.4 International collaborative study

The international collaborative study (EU-RL GMFF step 4) involved twelve laboratories, all being "National reference laboratories, assisting the CRL for testing and validation of methods for detection", as listed in annex to Regulation (EC) No 1981/2006. The study was carried out in accordance with the following internationally accepted guidelines:

The IUPAC "Protocol for the design, conduct and interpretation of method-performance studies" (Horwitz, 1995)
ISO 5725 (1994)

The objective of the international collaborative study was to verify in experienced laboratories the trueness and reproducibility of the PCR analytical method described under 3.2, above, that was provided by the applicant.

3.4.1 List of participating laboratories

The participants in the FG72 validation study were randomly selected, using a validated software, from within the 35 NRL that offered to participate.

Clear guidance was given to the selected laboratories with regards to the standard operational procedures to follow for the execution of the protocol (the report of the Validated Method is available at, <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>). The participating laboratories are listed in Table 4.

Table 4. Laboratories participating in the validation of the detection method for soybean FG72

Laboratory	Country
Bavarian Health and Food Safety Authority	DE
Environment Agency Austria	AT
Food and Environment Research Agency	UK
Genetically Modified Organism Controlling Laboratory	PL
Institute for Consumer Protection, Department 3 - Food Safety - Halle	DE
LGC Limited	UK
National Centre for Food, Spanish Food Safety Agency and Nutrition	ES
National Centre of Public Health Protection - Bulgarian National Laboratory for Genetically Modified Food	BG
Plant Health Laboratory	FR
Science and Advice for Scottish Agriculture (SASA)	UK
State Office for Agriculture, Food Safety and Fisheries	DE
Walloon Agricultural Research Centre (CRA-W)	BE

3.4.2 Real-time PCR equipment used in the study

Laboratories involved in the collaborative study used the following real-time PCR equipment: six laboratories used the ABI 7500, four used the ABI 7900, one used the ABI 7300 and one used the Stratagene Mx 3005.

3.4.3 Materials used in the international collaborative study

For the validation of the quantitative event-specific method, control samples were provided by the EU-RL GMFF to the participating laboratories. They consisted of:

- i) genomic DNA extracted from homozygous soybean seeds harbouring the event FG72, and
- ii) genomic DNA extracted from conventional soybean seeds genetically similar to those harbouring the FG72 event.

The control samples were prepared by the EU-RL GMFF from the samples provided by the applicant in accordance to Regulation (EC) No 1829/2003, Art 2.11⁵.

⁵ Control sample defined as the GMO or its genetic material (positive sample) and the parental organism or its genetic material that has been used for the purpose of the genetic modification (negative sample). Reg. (EC) No 1829/2003, Art. 2 (11)

These positive and negative control samples were used by the EU-RL GMFF to prepare standard and test samples (of unknown GM-content), containing mixtures of FG72 soybean and non-GM soybean, as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes. Given the ratio of 1 between GM and non-GM targets in the positive control sample, the same concentration levels of standards and test samples are equally expressed in terms of DNA copy number ratios or DNA mass fractions.

The calibration sample S1 was prepared by mixing the appropriate amount of FG72 DNA in control non-GM soybean DNA to obtain a 10% GM FG72. Calibration samples S2-S5 were prepared by 4-fold dilution from the S1 sample.

The 12 NRLs participating in the validation study received the following materials:

- ✓ Five calibration samples with known concentrations of GM-events (175 µL of DNA solution each) labelled from S1 to S5 (Table 1).
- ✓ Twenty blinded test DNA samples (87.5 µL of DNA solution each) labelled from U1 to U20, representing 5 GM levels (Table 5).

Table 5. FG72 GM contents in genome copy number and DNA mass

FG72 GM%
GM copy number/soybean genome copy number x 100
8.00
3.00
0.90
0.40
0.10

- ✓ Reaction reagents:
 - Universal PCR Master Mix (2x), one vial: 15 mL
 - distilled sterile water, one vial: 10 mL
- ✓ Primers and probes (1 tube each) as follows:
 - /e1* taxon-specific assay
 - KVM164 (10 µM): 160 µL
 - KVM165 (10 µM): 160 µL
 - TM021 (10 µM): 160 µL
 - FG72 assay provided by the applicant
 - MAE071 (10 µM): 320 µL
 - SHA097 (10 µM): 320 µL
 - TM325 (10 µM): 160 µL

3.4.4 Design of the collaborative study

Twenty test samples (labelled from U1 to U20), representing five GM levels, each in 4 replicates, were included in the validation study (Table 3). The detailed design of the PCR plates and other details were communicated to the participating laboratory in a validation protocol. On each PCR plate, the samples were analysed for the FG72 specific system and for the *le1* taxon-specific system. In total, two plates were run per participating laboratory. Participants determined the GM% according to the instructions provided) and using the excel sheet provided.

Each participating laboratory received the above described materials and prepared the master-mixes for the FG72 and lection assay in accordance with the description provided in the validation protocol. Calibration and test samples were loaded on the PCR plates as per recommended plate lay-out. The amplification reactions followed the cycling program provided. Raw data were reported according to instructions provided on an excel sheet designed, validated and distributed to participating laboratories by the EU-RL GMFF. The original data recorded in the excel sheets were provided to the EU-RL GMFF and back-up copies on CDs were subsequently delivered via mail. All the data received are stored by the EU-RL GMFF on a dedicated and protected server.

The EU-RL GMFF then analysed the data against parameters and limits set by the ENGL: trueness, precision, amplification efficiency and linearity

3.4.5 Deviations reported from the protocol

Nine laboratories reported no deviations from the method protocol

One laboratory sealed the plate with optical cap strips (Applied Biosystems, part number 4323032) instead of adhesive covers.

One laboratory found one of the test tubes empty (U14), corresponding to one replicate for GM-level 8.0%.

Another laboratory, due to pipetting error, could not return the value for U19, corresponding to one replicate for GM-level 3.0%.

4. Results

4.1 EU-RL GMFF experimental testing

Results of the EU-RL GMFF experimental testing were already reported, in chapter 2.

4.1.1 Determination of the zygoty ratio in the positive control sample

The results of the tests to determine the zygoty ratio in the positive control sample are shown in Table 6.

Table 6. Summary of dPCR analysis conducted on the FG72 and *Le1* targets in the positive control sample.

Mean ratio (FG72/<i>Le1</i>)	0.976
Standard deviation	0.064
RSD_r (%)	6.6
Standard error of the mean	0.017
Upper 95% CI of the mean	1.011
Lower 95% CI of the mean	0.941

In conclusions, the 95% confidence interval (CI) spans around 1 and therefore the mean ratio is not significantly different from an expected ratio of 1, assuming a GM homozygous and a single-copy reference target, for an alpha = 0.05.

Hence, given the above experimental data, in the positive control sample provided by the applicant in support to the validation of the method for detection of FG72 soybean the GM% expressed in GM- DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes equals the GM% expressed in DNA mass fraction:

$$\text{GM \% in DNA copy number ratio} = \text{GM \% in mass fraction}$$

4.1.2 In-house verification of method performance against ENGL method acceptance criteria

Test samples with GM-levels 8.0%, 3.0%, 0.9% and 0.4% were tested in two real-time PCR runs (run A and B) with two replicates for each GM-level on each plate (total of four replicates per GM-level). The test sample with GM-level 0.1% was tested in 15 replicates in one run (run C). Results are shown in Table 7 and 8.

Table 7. Standard curve parameters

	FG72-system			Lec reference system		
	Slope	PCR efficiency*	R ²	Slope	PCR efficiency*	R ²
Run A	-3.377	97.8	0.999	-3.321	100.1	0.997
Run B	-3.250	103.1	0.998	-3.412	96.4	0.999
Run C	-3.404	96.7	0.998	-3.292	101.3	0.995

* PCR efficiency (%) is calculated using the formula $\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$

According to the ENGL method acceptance criteria, the average value of the slope of the standard curve shall range from -3.1 to -3.6 and the R² coefficient shall be ≥ 0.98 .

Table 7 documents that the slope of the standard curve, and the R² coefficient were within the limits established by the ENGL.

Table 8. Outcome of the in-house verification, with regards to the quantification of the five test samples

GM-levels %	Measured GM %	Bias %	Precision (RSDr %)
8.0	8.56	6.9	1.2
3.0	2.96	-1.3	11.1
0.9	0.85	-5.5	11.2
0.4	0.38	-5.3	4.6
0.1	0.10	-0.5%	18%

According to the ENGL method acceptance criteria, the method trueness (measured as bias %) should be within $\pm 25\%$ of the accepted reference value over the dynamic range. The method's precision as RSDr % should be $\leq 25\%$ over the dynamic range. Table 8 documents that trueness (estimated through bias %) and precision (estimated through RSDr, relative standard deviation of repeatability) of quantification were within the limits established by the ENGL.

4.2 Results of the international collaborative study

4.2.1 PCR efficiency and linearity

Standard curve slopes [from which the PCR efficiency (%) is calculated using the formula $\text{Efficiency} = (10^{(-1/\text{slope})} - 1) \times 100$, and R² values (expressing the linearity of the regression) reported by participating laboratories for the FG72 and the *le1* assays are reported in Table 9.

Table 9. Values of slope, PCR efficiency and R^2 obtained during the validation study

Lab	Plate	FG72			<i>le1</i>		
		Slope	PCR Efficiency (%)	R^2	Slope	PCR Efficiency (%)	R^2
1	A	-3.41	97	1.00	-3.44	95	1.00
	B	-3.35	99	1.00	-3.46	94	1.00
2	A	-3.42	96	1.00	-3.65	88	1.00
	B	-3.48	94	1.00	-3.54	91	1.00
3	A	-3.60	90	1.00	-3.77	84	1.00
	B	-3.44	95	1.00	-3.66	88	1.00
4	A	-3.53	92	0.99	-3.60	90	1.00
	B	-3.45	95	1.00	-3.64	88	1.00
5	A	-3.45	95	1.00	-3.47	94	1.00
	B	-3.37	98	1.00	-3.50	93	1.00
6	A	-3.44	95	1.00	-3.75	85	0.99
	B	-3.40	97	1.00	-3.85	82	1.00
7	A	-3.45	95	1.00	-3.57	91	1.00
	B	-3.47	94	1.00	-3.55	91	1.00
8	A	-3.42	96	1.00	-3.68	87	1.00
	B	-3.40	97	1.00	-3.58	90	1.00
9	A	-3.49	94	1.00	-3.55	91	1.00
	B	-3.45	95	1.00	-3.48	94	1.00
10	A	-3.56	91	1.00	-3.56	91	1.00
	B	-3.43	96	1.00	-3.52	92	1.00
11	A	-3.58	90	1.00	-3.73	85	1.00
	B	-3.50	93	1.00	-3.59	90	1.00
12	A	-3.60	90	1.00	-3.65	88	1.00
	B	-3.50	93	1.00	-3.60	90	1.00
Mean		-3.47	94	1.00	-3.60	90	1.00

Table 9 indicates that the efficiency of amplification for the FG72 system ranges from 90 to 99% with a linearity of 1.00 and the amplification efficiency for the soy-specific reference system ranges from 82% to 95% with a linearity of 1.00. The mean PCR efficiency was 94% for the FG72 assay and 90% for the *le1* assay, with both values within the ENGL acceptance criteria. The R^2 of the methods was 1.00 both for FG72 and *le1* assays.

The results confirm the appropriate performance characteristics of the method tested in terms of efficiency and linearity.

4.2.2 GMO quantification

Table 10 reports the mean values of the four replicates for each GM level as provided by all laboratories, before application of the Cochran and Grubbs tests for identification of outlying values, as per ISO 5725.

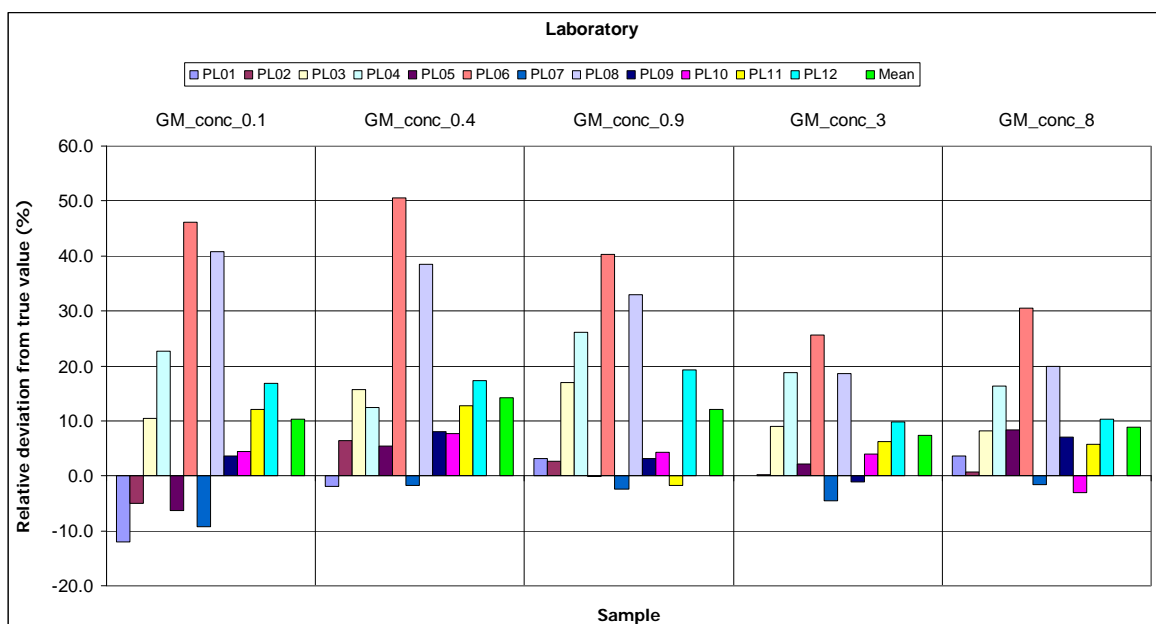
Table 10. GM% mean values determined by laboratories for test samples

GMO content $\text{GMO\%} = \text{GMO copy number} / \text{soybean genome copy number} \times 100$ and $\text{GM DNA mass} / \text{soybean DNA mass} \times 100$																				
LAB	0.1				0.4				0.9				3.0				8.0			
	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4	REP 1	REP 2	REP 3	REP 4
1	0.09	0.11	0.07	0.09	0.45	0.34	0.39	0.39	0.93	0.92	0.87	0.99	1.98	2.94	2.82	4.26	10.19	7.78	7.93	7.27
2	0.09	0.10	0.08	0.11	0.43	0.43	0.39	0.46	0.86	0.84	1.04	0.95	2.74	-	3.25	3.03	7.95	8.07	8.24	7.99
3	0.11	0.11	0.10	0.12	0.44	0.48	0.47	0.46	0.97	1.02	1.24	0.99	3.07	3.68	3.18	3.15	8.99	8.84	8.49	8.29
4	0.14	0.11	0.14	0.11	0.61	0.39	0.42	0.38	1.30	1.12	1.03	1.09	3.67	4.13	3.53	2.93	9.96	10.56	7.67	9.04
5	0.10	0.08	0.10	0.10	0.42	0.46	0.39	0.42	0.91	0.84	0.87	0.98	3.10	3.10	3.13	2.94	8.93	9.63	7.17	8.93
6	0.13	0.14	0.14	0.18	0.58	0.66	0.69	0.49	1.04	1.16	1.65	1.20	4.23	4.30	3.58	2.97	14.83	11.21	6.23	9.51
7	0.09	0.09	0.09	0.09	0.40	0.39	0.38	0.40	0.89	0.79	0.93	0.91	2.80	2.76	3.23	2.67	7.78	7.95	7.97	7.81
8	0.17	0.13	0.12	0.14	0.52	0.66	0.43	0.61	1.03	1.14	1.38	1.25	3.71	3.76	3.65	3.11	9.99	10.25	8.41	9.74
9	0.10	0.10	0.10	0.11	0.44	0.42	0.43	0.43	0.90	0.91	1.00	0.90	3.15	2.70	3.05	2.97	-	8.36	8.41	8.91
10	0.12	0.10	0.10	0.10	0.42	0.39	0.45	0.46	0.91	0.92	1.01	0.91	3.00	2.98	3.46	3.02	7.84	7.77	7.85	7.55
11	0.12	0.10	0.11	0.12	0.45	0.38	0.50	0.46	0.86	0.82	0.86	0.99	3.24	3.36	3.15	3.00	7.96	10.26	7.50	8.11
12	0.12	0.12	0.11	0.12	0.46	0.46	0.44	0.51	1.00	1.03	1.16	1.11	3.16	3.32	3.43	3.26	9.50	9.68	7.94	8.18

Table 10 indicates that most of the single measurements per laboratory (replicates) showed that trueness was within the ENGL limits. At 0.1% GM level, thirty-eight replicate values out of forty-eight were within the 25% upper limit of quantification (bias %), forty at the 0.4% GM level, thirty-nine at the 0.9% GM level, forty-two at the 3.0% GM level, forty-two at the 8.0% GM-level. Data of all the replicates were retained to feed the statistical analysis whose results are reported in Table 11.

In Figure 1 the relative deviation from the true value for each GM level tested is shown for each laboratory. The coloured bars represent the deviation of the GM level measured by the respective laboratory in % of the true GM level; the green bar on the right represents the mean relative deviation over all 12 participating laboratories for each true GM level.

Figure 1. Relative deviation (%) from the true value of FG72 for all laboratories*



*PL05 at GM level 0.9%, PL 01 and 02 at GM level 3.0% had very small relative deviations from the true value and the corresponding histograms do not show up in Figure 1.

Overall, most mean relative deviations from the true values are within the ENGL acceptance criterion of maximum 25% for all GM levels, in particular, 10 laboratories were within the limits at the 0.1% GM-level, 10 at the 0.4%, 9 at the 0.9%, 11 at the 3.0% and 11 at the 8.0%. Two laboratories overestimated the DNA content of sample 0.1% by more than 40% and one of those laboratories also overestimated the DNA content of samples 0.4 and 0.9% by almost 40% and over 30% respectively. All the data were retained to feed the statistical analysis whose results are reported in Table 11.

The bias generated by all laboratories is about +10% or below, i.e. the method is slightly over-predictive but well within the accepted limits.

5. Method performance requirements

Among the performance requirements established by ENGL and adopted by the EU-RL GMFF (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>), repeatability and reproducibility are to be assessed through an international collaborative trial, carried out with the support of twelve ENGL laboratories (see Table 3). Table 11 illustrates the estimation of repeatability and reproducibility at the various GM levels tested during the collaborative trial.

According to the ENGL method performance requirements, the relative reproducibility standard deviation (RSD_R), that describes the inter-laboratory variation, should be below 35% at the target

concentration and over the majority of the dynamic range, while it should be below 50% at the lower end of the dynamic range.

As it can be observed in Table 11, the method satisfies this requirement at all GM levels tested. In fact, the highest value of RSD_R is 20% at the 0.1% GM level, thus within the acceptance criterion.

Table 11. FG72, summary of validation results expressed as GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

	Test Sample Expected GMO %				
	0.1	0.4	0.9	3.0	8.0
Laboratories having returned valid results	12	12	12	12	12
Samples per laboratory	4	4	4	4	4
Number of outliers	0	1	1	1	1
Reason for exclusion	-	DG	C	C	C
Mean value	0.11	0.44	0.99	3.24	8.55
Relative repeatability standard deviation, RSD_r (%)	12	12	9	10	10
Repeatability standard deviation	0.01	0.05	0.09	0.31	0.84
Relative reproducibility standard deviation, RSD_R (%)	20	14	14	12	11
Reproducibility standard deviation	0.02	0.06	0.13	0.39	0.92
Bias (absolute value)	0.010	0.044	0.086	0.242	0.549
Bias (%)	10.4	11.0	9.5	8.1	6.9

C= Cochran's test, DG= Double Grubbs; identification and removal of outliers through Cochran and Grubbs tests, according to ISO 5725-2. Bias is estimated according to ISO 5725 data analysis protocol.

Table 11 also documents the relative repeatability standard deviation (RSD_r) estimated for each GM level. In order to accept methods for collaborative study, the EU-RL GMFF requires that the RSD_r value is below 25%, as indicated by the ENGL (Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing" <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>). As it can be observed from the values reported, the method showed a repeatability standard deviation below 25% at all GM levels, with the highest value of RSD_r (%) of 12% at 0.1% and 0.4% GM level.

The trueness of the method is estimated using the measures of the method bias for each GM level. According to ENGL method performance requirements, trueness should be $\pm 25\%$ across the entire dynamic range. In this case, the method satisfies this requirement across the dynamic range tested, with the highest value of bias (%) of 11% at the 0.4% GM level.

6. Compliance of the method of detection of event FG72 with the requirements of Regulation (EU) No 619/2011

To verify the compliance of the method under validation with the requirements of Regulation (EU) No 619/2011, the following steps were carried out and their outcome is summarised in Table 12:

- at step 2 of the validation process (scientific assessment of the dossier), the EU-RL GMFF concluded that it could accept the applicant's data on method performance and therefore accepted that the RSDr at the level of 0.08% in terms of GM- DNA copy numbers in relation to target taxon specific DNA copy numbers was 15% on 18 replicates (Table 4), hence below 25%;
- at step 3 of the validation process (in-house testing of the method), the EU-RL GMFF determined the RSDr at the level of 0.1% related to mass fraction of GM-material by fifteen replicates in repeatability conditions (single run). The RSDr was 18% (Table 8), hence within the range indicated by the applicant;
- further to the conclusion of step 4 of the validation process (ring trial), the EU-RL GMFF analysed the data generated by the 12 participating laboratories for determining the method performance parameters. It found that the RSDr over all laboratories of the method at the level of 0.1% related to mass fraction of GM-material was 12%.

Table 12. Precision of the event-specific method for quantitative detection of FG72 at or around 0.1% level related to mass fractions of GM material

Source	RSDr %	GM %
Applicant' method optimisation*	15 %	0.08 %
EU-RL GMFF tests	18 %	0.1 %
Collaborative study	12 %	0.1 %

* GM- DNA copy numbers in relation to target taxon specific DNA copy numbers

Based on the results of the EU-RL GMFF in-house verification and of the collaborative study, it is concluded that the method RSDr is equal or less than 25% at the level of 0.1% related to mass fraction of GM-material, hence the method for quantitative detection of event FG72 soybean meets the requirement laid down in Regulation (EU) No 619/2011.

7. Conclusion

The method provided by the applicant and described in detail under 3.2 (available at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>) has been validated in accordance to the EU-RL GMFF validation scheme; respecting all requirements of the relevant EU legislation and international standards.

The dossier was found complete (step 1) and the scientific dossier analysis (step 2) concluded that the method met the ENGL minimum performance criteria for a quantitative PCR method for detection and quantification of GM events for entering into validation.

The subsequent in-house verification of the method (step 3) by the EU-RL GMFF confirmed this conclusion.

The international collaborative study (step 4) yielded data that also indicated that the method meets all acceptance criteria and method performance requirements recommended by the ENGL (as detailed at <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) for a valid quantitative PCR method for detection and quantification of GM-events.

In conclusion, the method is considered applicable to the control samples provided by the applicant (see paragraph 3.4.3), in accordance with the requirements of Annex I-2.C.2 to Commission Regulation (EC) No 641/2004 and (EU) No 619/2011. The method is also applicable to appropriately extracted soybean DNA

8. References

1. Horwitz W., 1995. Protocol for the design, conduct and interpretation of method performance studies, *Pure and Appl. Chem.* 67, 331-343.
2. International Standard (ISO) 5725, 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardization, Genève, Switzerland.
3. Arumuganathan K. and Earle E. D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.



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Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit



Event-specific Method for the Quantification of Soybean Event FG72 Using Real-time PCR

Protocol

16 July 2012

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Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit**

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1. General information and summary of the methodology

This protocol describes an event-specific real-time quantitative TaqMan® PCR (polymerase chain reaction) procedure for the determination of the relative content of soybean event FG72 DNA to total soybean DNA in a sample.

Template DNA extracted by means of suitable methods should be tested for quality and quantity prior to use in PCR assay. Tests for the presence of PCR inhibitors (e.g. monitor run of diluted series, use of DNA spikes) are recommended.

For the specific detection of soybean event FG72, a 70-bp fragment of the region spanning the 3' insert-to-plant junction in soybean FG72 event is amplified using specific primers. PCR products are measured during each cycle (real-time) by means of a target-specific oligonucleotide probe labelled with the fluorescent dye FAM (6-carboxyfluorescein), as a reporter at its 5' end, and MGB-NFQ (minor groove binding non-fluorescent quencher) at its 3' end.

For the relative quantification of soybean event FG72 DNA, a soybean-specific reference system amplifies a 102-bp fragment of *lectin1* (*le1*), a soybean endogenous gene (Accession number, GeneBank: K00821) using *le1* gene-specific primers and a *le1* gene-specific probe labelled with VIC as reporter dye at its 5' end, and TAMRA (6-carboxytetramethylrhodamine) as quencher at its 3' end.

The measured fluorescence signal passes a threshold value after a certain number of cycles. This threshold cycle is called the "Ct" value. For quantification of the amount of FG72 DNA in a test sample, Ct values for the FG72 and *le1* systems are determined for the sample. Standard curves are then used to estimate the relative amount of FG72 DNA to total soybean DNA.

2. Validation and performance characteristics

2.1 General

The method was optimised for suitable DNA extracted from mixtures of genetically modified and conventional soybean seeds. The reproducibility and trueness of the method were tested through an international collaborative ring trial using DNA samples at different GM contents.

2.2 Collaborative trial

The method was validated in an international collaborative study by the EU- RL GMFF. The study was undertaken with twelve participating laboratories in August-September 2011.

Each participant received twenty blind samples containing soybean FG72 genomic DNA at five GM contents, ranging from 0.1% to 8%.

Each test sample was analysed by PCR in three repetitions. The study was designed as a blind quadruplicate collaborative trial; each laboratory received each GM level of event FG72 in four replicates. Two replicates of each GM level were analysed on the same PCR plate.

A detailed validation report can be found at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

2.3 Limit of detection (LOD)

According to the method developer, the relative LOD of the method is at least 0.023% in 200 ng of total soybean DNA. The relative LOD was not assessed in the collaborative study.

2.4 Limit of quantification (LOQ)

According to the method developer, the relative LOQ of the method is at least 0.08% in 200 ng of total soybean DNA. The lowest relative GM content of the target sequence included in the collaborative trial was 0.1%.

2.5 Molecular specificity

According to the method developer, the method exploits a unique DNA sequence in the region spanning the 3' insert-to-plant junction in soybean FG72; the sequence is specific to event FG72 and thus imparts event-specificity to the method.

The specificity of the soybean taxon-specific assay was assessed by the method developer in real-time PCR using 200 ng of conventional genomic DNA extracted from soybean, rice, cotton, oilseed rape and maize. According to the method developer the soybean-specific reference system did not react with any target DNA except the positive control.

The specificity of the event-specific assay was assessed by the applicant in real-time PCR using genomic DNA (50 ng) extracted from conventional soybean and soybean FG72 event as positive control sample and, rice LLRICE62, oilseed rape (OSR) MS1, MS8, RF1, RF2, RF3, Topas19-2, T45, OXY-235, RT73; soybean A2704-12, A5547-127, GTS40-3-2; Cotton LLCotton25, GHB614, T304-40, GHB119, MON1445; maize MON810, BT11, GA21, NK603, T25.

According to the method developer, apart from the positive control reaction, the forward and reverse oligonucleotide primers and the Taqman probe of the FG72 event showed no amplification signals following quantitative PCR analysis (45 cycles).

3. Procedure

3.1 General instructions and precautions

- The procedures require experience of working under sterile conditions.
- Laboratory organisation, e.g. "forward flow direction" during PCR-setup, should follow international guidelines, e.g. ISO 24276:2006.

- PCR reagents should be stored and handled in a separate room where no nucleic acids (with exception of PCR primers or probes) or DNA degrading or modifying enzymes have been handled previously. All handling of PCR reagents and controls requires dedicated equipment, especially pipettes.
- All the equipment used should be sterilised prior to use and any residue of DNA has to be removed. All material used (e.g. vials, containers, pipette tips, etc.) must be suitable for PCR and molecular biology applications. They must be DNase-free, DNA-free, sterile and unable to adsorb protein or DNA.
- Filter pipette tips protected against aerosol should be used.
- Powder-free gloves should be used and changed frequently.
- Laboratory benches and equipment should be cleaned periodically with 10% sodium hypochloride solution (bleach).
- Pipettes should be checked regularly for precision and calibrated, if necessary.
- All handling steps, unless specified otherwise, should be carried out at 0 – 4°C.
- In order to avoid repeated freeze/thaw cycles aliquots should be prepared.

3.2 Real-time PCR for quantitative analysis of soybean event FG72

3.2.1 General

The PCR set-up for the taxon-specific target sequence (*le1*) and for the GMO (event FG72) target sequence is carried out in separate vials. Multiplex PCR (using differential fluorescent labels for the probes) has not been tested or validated.

The method is developed for a total volume of 25 µL per reaction mixture with the reagents as listed in Table 1 and Table 2.

3.2.2 Calibration

The calibration curves consist of five samples. The first point of the calibration curves is a sample containing 10% soybean FG72 DNA in a total of 150 ng of soybean DNA (corresponding to approximately 132743 soybean genome copies with one genome assumed to correspond to 1.13 pg of haploid soybean genomic DNA)⁽¹⁾. The other four standards are prepared by serial 4-fold dilution of the 10% standard.

A calibration curve is produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. This can be done e.g. by means of spreadsheet

software, e.g. Microsoft Excel, or directly by options available with the sequence detection system software.

The copy number measured for each unknown sample DNA is obtained by interpolation from the standard curves.

3.2.2 Real-time PCR set-up

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.
2. To prepare the amplification reaction mixtures add the following components (Table 1 and 2) in two reaction tubes (one for the FG72 assay and one for the *le1* assay) on ice in the order mentioned below (except DNA).

Table 1. Amplification reaction mixture in the final volume/concentration per reaction well for the FG72 assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x) with UNG	1x	12.5
MAE071 (10 µM)	400 nM	1.0
SHA097 (10 µM)	400 nM	1.0
TM325 (10 µM)	200 nM	0.5
Nuclease free water	#	5.0
Template DNA (100 ng)	#	5.0
Total reaction volume:		25 µL

Table 2. Amplification reaction mixture in the final volume/concentration per reaction well for the soybean *le1* assay.

Component	Final concentration	µL/reaction
TaqMan® Universal PCR Master Mix (2x) with UNG	1x	12.5
KVM164 (10 µM)	200 nM	0.5
KVM165 (10 µM)	200 nM	0.5
TM021 (10 µM)	200 nM	0.5
Nuclease free water	#	6.0
Template DNA (100 ng)	#	5.0
Total reaction volume:		25 µL

3. Mix well and centrifuge briefly.

4. Prepare two reaction tubes (one for the soybean FG72 and one for the *le1* system) for each DNA sample to be tested (standard curve samples, unknown samples and control samples).
5. Add to each reaction tube the correct amount of reaction mix for 3.5 PCR repetitions (e.g. 70 μ L for the *le1* reference system and 70 μ L for the FG72 soybean system). Add to each tube the correct amount of DNA for 3.5 PCR repetitions (e.g. 17.5 μ L DNA). The additional 0.5 repetition included will ensure adequate volume when loading the samples. Vortex each tube for approx. 10 sec. This step is mandatory to reduce to a minimum the variability among the repetitions of each sample.
6. Spin down the tubes in a micro-centrifuge. Aliquot 25 μ L in each well. Seal the reaction plate with optical cover or optical caps. Centrifuge the plate at low speed (e.g. approximately 250 x *g* for 1 minute at 4°C to room temperature) to spin down the reaction mixture.
7. Place the plate into the instrument.
8. Run the PCR with the cycling program described in Table 3.

Table 3. Cycling program for FG72/*le1* assays.

Step	Stage	T (°C)	Time (s)	Acquisition	Cycles
1	UNG*	50	120	No	1X
2	Initial denaturation	95	600	No	1X
3	Amplification	Denaturation	95	15	No
		Annealing & Extension	60	60	Yes
					45X

*UNG: Uracil-N-glycosylase

3.3 Data analysis

After the real-time PCR, analyse the run following the procedure below:

- a) Set the threshold: display the amplification curves of one assay (e.g. FG72) in logarithmic mode. Locate the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR) and where there is no "fork effect" between repetitions of the same sample. Press the "update" button to ensure changes affect Ct values (only needed for some analysis software). Switch to the linear view mode by clicking on the Y axis of the amplification plot and check that the threshold previously set falls within the exponential phase of the curves.
- b) Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25, set the baseline crossing at Ct = 25 – 3 = 22).
- c) Save the settings.
- d) Repeat the procedure described in a), b) and c) on the amplification plots of the other system (e.g. *le1*).

e) Save the settings and export all the data to a text file for further calculations.

3.4 Calculation of results

After having defined a threshold value within the logarithmic phase of amplification as described above, the instrument's software calculates the Ct-values for each reaction.

The standard curves are generated both for the *le1* and the FG72 specific assays by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a linear regression line into these data.

Thereafter, the standard curves are used to estimate the DNA copy numbers in the unknown sample.

To obtain the percentage value of event FG72 DNA in the unknown sample, the FG72 copy number is divided by the copy number of the soybean reference gene (*le1*) and multiplied by 100 ($GM\% = FG72/Le1 \times 100$).

4. Materials

4.1 Equipment

- Real-time PCR instrument for plastic reaction vessels (glass capillaries are not recommended for the described buffer composition)
- Plastic reaction vessels suitable for real-time PCR instrument (enabling undisturbed fluorescence detection)
- Software for run analysis (mostly integrated in the software of the real-time PCR instrument)
- Microcentrifuge
- Micropipettes
- Centrifuge for PCR-plates
- Vortex
- Rack for reaction tubes
- 0.2/1.5/2.0 mL reaction tubes

4.2 Reagents

- TaqMan® Universal PCR Master Mix (2X). Applied Biosystems Part No 4326708.

4.3 Primers and Probes

Oligonucleotides	Name	DNA Sequence (5' to 3')	Length (nt)
FG72			
Forward primer	MAE071	5' AgA TTT gAT Cgg gCT gCA gg 3'	20
Reverse primer	SHA097	5' gCA CgT ATT gAT gAC CgC ATT A 3'	22
Probe	TM325	6-FAM-5' AAT gTg gTT CAT CCg TCT T-MGBNFQ-3'	19
<i>le1</i>			
Forward primer	KVM164*	5' CTT TCT CgC ACC AAT TgA CA 3'	20
Reverse primer	KVM165	5' TCA AAC TCA ACA gCg ACg AC 3'	20
Probe	TM021	6-VIC®-5' CCA CAA ACA CAT gCA ggT TAT CTT gg-TAMRA-3'	26

FAM: 6-carboxyfluorescein; MGBNFQ: Minor Groove Binder/Non-Fluorescent Quencher; TAMRA: carboxytetramethylrhodamine;

NOTE. The same primer name KVM164 is attributed by Bayer CropScience to two different primer sequences used as forward primer in the reference system for relative quantification of soybean events A2704-12, A5547-127 and FG72.

The difference lies in an additional triplet 'CAC' at the 5' end of the KVM164 applied for the relative quantification of soybean event A2704-12 (KVM164: CAC CTT TCT CgC ACC AAT TgA CA, 23bp long, http://gmo-crl.jrc.ec.europa.eu/summaries/A2704-12_soybean_validated_Method.pdf). The CAC triplet is not present in the KVM164 primer used in the reference system for relative quantification of soybean events A5547-127 (http://gmo-crl.jrc.ec.europa.eu/summaries/A5547_validated%20Method.pdf) and FG72

5. References

1. Arumuganathan K., Earle E.D., 1991. Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter*, 9: 208-218.

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Abstract

In line with its mandate¹ the European Union Reference Laboratory for GM Food and Feed (EU-RL GMFF), in collaboration with the European Network of GMO Laboratories (ENGL), has validated an event-specific PCR method for detecting and quantifying soybean event FG72 (unique identifier MST-FGØ72-2). The validation study was conducted according to the EU-RL GMFF validation procedure [<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>] and internationally accepted guidelines², involving 12 laboratories.

In accordance with current EU legislation¹, Bayer CropScience has provided the detection method and the samples (genomic DNA from soybean seeds harbouring the FG72 event as positive control DNA, genomic DNA from conventional soybean seeds as negative control DNA). The EU-RL GMFF prepared the validation samples (calibration samples and blind samples at test GM percentage [DNA/DNA]), organised an international collaborative study and analysed the results.

The study confirms that the method meets the method performance requirements as established by the EU-RL and the ENGL detailing the provisions of Annex I-2.C.2 to Regulation (EC) No 641/2004 and it fulfils the analytical requirements of Regulation (EU) No 619/2011.

This report is published at <http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm>.

¹ Regulation (EC) No 1829/2003 of 22 September 2003 “on genetically modified food and feed” and with Regulation (EC) No 641/2004 of 6 April 2004 “on detailed rules for the implementation of Regulation (EC) No 1829/2003”,

² The IUPAC “Protocol for the design, conduct and interpretation of method-performance studies” (Horwitz, 1995); and ISO 57: (1994).

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

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